Prognostic Impact of Combined Fludarabine, Treosulfan and Mitoxantrone Resistance Profile in Childhood Acute Myeloid Leukemia

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Abstract. *Background: The role of cellular drug resistance in childhood acute myeloid leukemia (AML) has not yet been established. The aim of the study was the analysis of the clinical value of ex vivo drug resistance in pediatric AML. Patients and Methods: A cohort of 90 children with de novo AML were assayed for drug resistance profile by the 3-4,5-dimethylthiazol-2-yl-2,5-difenyl tetrazolium bromide (MTT) assay and prognostic model of in vitro drug sensitivity was analyzed. Results: Children who relapsed during follow-up showed higher in vitro resistance of leukemic blasts to most of the drugs tested, except for cytarabine, cladribine, vincristine, mercaptopurine and thioguanine. A combined in vitro drug resistance profile to fludarabine, treosulfan and mitoxantrone (FTM score) was defined and it had an independent prognostic significance for disease free survival in pediatric AML. Conclusion: The combined fludarabine, treosulfan and mitoxantrone resistance profile to possibly may be used for better stratification of children with AML or indicate the necessity for additional therapy.*

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The response to therapy in childhood acute myeloid leukemia (AML) is much worse than in acute lymphoblastic leukemia (ALL). In contrast to ALL, optimal risk-group stratification so far has not been achieved in childhood AML (1, 2). Favorable cytogenetics and early response to therapy are regarded as the most important prognostic factors, however their value is still limited (3-8). Unlike ALL (9-11), the role of cellular drug resistance in childhood AML has not yet been established. Several groups reported possible the prognostic value of *in vitro* drug sensitivity in pediatric AML, showing a good correlation between *in vitro* drug resistance and short-term clinical outcome after chemotherapy (12-16). These findings were related mainly to cytarabine (15) and cyclophosphamide (16). Part of these studies included both children and adults. Recently published large studies showed no correlation between *in vitro* drug

resistance to individual drugs and long-term clinical outcome in childhood AML (17-19). So far, no data exist to support the prognostic value of any *in vitro* drug resistance profile in childhood AML, while this relationship has been confirmed in adult AML (20). In our previous report, we showed the possible prognostic value of a combined fludarabine, treosulfan and etoposide resistance profile to in a subgroup of children with AML undergoing hematopoietic stem cell transplantation (21).

We conducted a study to compare the response to therapy with the drug resistance profile in a large group of children with AML by evaluating the *in vitro* sensitivity profile based on a combination of chemosensitivity to different drugs.

Patients and Methods

Patients. A total of 90 children aged 0.1-17.8 years (median 9.3 years), with *de novo* AML were initially included in the study. The median value of their initial white blood cell count was 16.5×10^9/L (range, 0.5-516×10^9/L) in this group. French-American-British (FAB) morphology was diagnosed as: M0 in 6 children, M1 in 16, M2 in 24, M3 in 12, M4 in 15, M5 in 10, M6 in 5 and M7 in 2 patients. All patients were treated according to the AML-PPLLSG-98 protocol of the Polish Pediatric Leukemia and Lymphoma Study Group (6). Patients with secondary AML, Down syndrome, biphenotypic leukaemia or death before treatment were not included in the study. The study was approved by the local Ethics Committee and written informed consent was obtained from all patients and their parents.

Drugs. Drug resistance profiling was carried out using the 3-4,5-dimethylthiazol-2-yl-2,5-difenyl tetrazolium bromide (MTT) assay for 3-30 drugs for each patient. The following drugs and concentrations were used: prednisolone (Jelfa, Jelenia Gora, Poland; concentration range 0.02-694 μM), dexamethasone (Jelfa; 0.5 nM-15.3 μM), daunorubicin (Daunorubicin; Rhone-Poulenc-Rhorer, Paris; 0.002-3.5 μM), doxorubicin (Doxorubicin; Farmitalia, Milan; 0.01-13.8 μM), idarubicin (Zavedos; Pharmacia & Upjohn, Milan; 0.003-3.7 μM), epirubicin (Farmorubicin; Farmitalia, Milan; 0.003-3.4 μM), mitoxantrone (Mitoxantrone; Jelfa; 0.002-1.9 μM), L-asparaginase (Medac, Hamburg, Germany; 0.0032–10 IU/L), vincristine (Oncovin; Eli-Lilly, Indianapolis, IN, USA; 0.02-21 μM), vindesine (Eli-Lilly; 0.03-30 μM), etoposide (Vepeside; Bristol-Myers Squibb, Princeton, NJ, USA; 0.08-85 μM), teniposide (Vumon; Bristol-Myers Squibb; 0.01-10 μM), 6-mercaptopurine (M7000; Sigma; St Louis, MO; 91-2937 μM), 6-thioguanine (A4882; sigma; 9.3-299 μM), cytarabine (Cytosar; Pharmacia & Upjohn, Bentley, Australia; 0.04-41 μM), fludarabine phosphate (Fludara; Schering AG, Berlin, Germany; 0.05-54 μM), cladribine (Biodribin; Bioton, Warsaw, Poland; 0.001-140 μM), 4-HOO-cyclophosphamide (Asta Medica AG, Frankfurt/Main; 0.3-341 μM), 4-HOO-ifosfamide (Asta Medica AG; 0.3-341 μM), glufosfamide (Asta Medica AG; 0.5-522 μM), mafosfamide (Asta Medica AG; 0.19-200 μM), melphalan (Glaxo Wellcome, Parma, Italy; 0.12-131 μM), thiotepa (Thiotepa; Lederle, Riemser, Greifswald, Germany; 0.16-528 μM), treosulfan (Ovastat; Medac; 0.002-3.6 μM), actinomycin D (Cosmogen, Merck Sharp & Dohme, Vienna, Austria; 0.03 nM-4 μM), cisplatin (Platidam; Pliva-Lachema, Brno, Czech Republic; 0.33-333 μM), carboplatin (Cycloplatin; Pliva-Lachema, Brno, Czech Republic; 1.34-1346 μM), pactitaxel (Taxol; Bristol-Myers Squibb; 0.05–58 μM), docetaxel (Taxotere; Rhone-Poulenc Rorer, Antony, France; 0.0001-12 μM), mitomycin C (Mitomycin; Kyowa Hakko, Tokyo; 0.03-30 μg/ml).

Table I. *Univariate risk factor analysis for pLFS.*

Parameter	Number of patients	pLFS	*p*-value
Age(years)			NS
<2	7	0.36±0.15	
2-10	38	0.42±0.08	
>10	45	0.53±0.07	
Gender			NS
Male	51	0.50±0.07	
Female	39	0.42±0.07	
FAB			0.034
M0	6	0.43±0.19	
M1	16	0.39±0.12	
M2	24	0.73±0.09	
M3	12	0.64±0.13	
M4	15	0.29±0.11	
M5	10	0.20±0.13	
M6	5	0.33±0.19	
M7	2		
WBC ($\times10^9$/L)			0.022
<20	51	0.47±0.07	
20-100	20	0.71±0.09	
>100	19	0.24±0.09	
Cytogenetics			0.055
Favorable*	7	1.00±0.00	
Other	30	0.74±0.09	
Early BM response			0.013
Yes	68	0.76±0.07	
No	20	0.54±0.13	
Remission by day 28			0.018
Yes	67	0.74±0.07	
No	15	0.35±0.26	
FTM score			0.034
Sensitive	37	0.73±0.12	
Resistant	37	0.50±0.14	

*Favorable blast karyotype was determined by t(8;21), t(15;17) or inv(16). FAB: French-American-British morphology classification; WBC: White blood cell count; BM: bone marrow; FTM: fludarabine, treosulfan and mitoxantrone.

In vitro drug resistance profile. Leukemic cells were separated on a Ficoll gradient and washed twice with RPMI-1640. The viability, recovery, cell morphology and percentage of blasts were analysed before and after the assay. Only samples with at least of 70% of myeloblasts which successfully tested for at least 3 drugs were included in the study. Cytotoxicity of tested compounds to leukemic cells was measured in duplicates by the MTT assay, as described elsewhere (21, 22). The cytotoxicity was expressed as the LC 50, the concentration lethal to 50% of the cells.

According to the median cytotoxicity for each of the tested drugs, all patients were scored as sensitive (score 1) or resistant (score 2) to this drug. Reference values for the combined *in vitro*

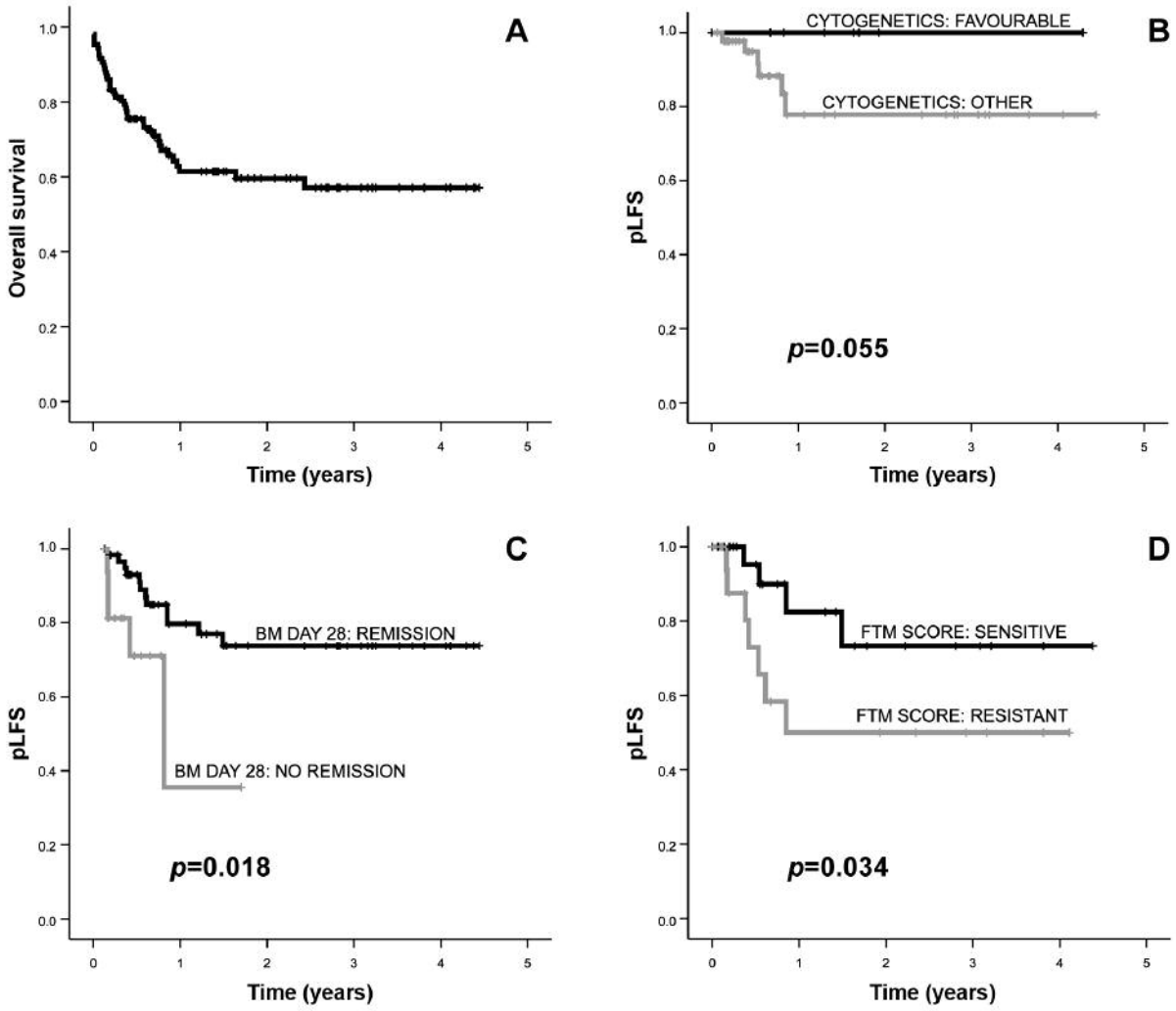

Figure 1. *Survival data for risk factors. A, Overall survival; B, probability of leukemia free survival (pLFS) by cytogenetics; C, pLFS by bone marrow (BM) response by day 28; D, pLFS by combined in vitro resistance profile to fludarabine, treosulfan and mitoxantrone (FTM score).*

resistance profile for fludarabine, treosulfan and mitoxantrone (FTM score) were determined based on the division of all LC 50 values into two equal groups based on the cut-off values created by the median. All patients were retrospectively re-assessed based on the results obtained over the whole group. The FTM score was defined as the sum of the three respective score values for fludarabine, treosulfan and mitoxantrone, thus FTM score ranged from 3 to 6. A sensitive FTM profile was regarded as a score of 3-4, while resistant as 5-6. Results were compared between groups of children with leukemia subtypes. The relative resistance (RR) between groups of patients for each drug was calculated as a ratio of median values of the LC 50 for this drug in both groups.

Definitions. Complete remission (CR) was defined as no more than 5% blasts in bone amrrow (BM) aspirate with signs of regeneration of normal hematopoiesis, no blasts in the peripheral blood and the disappearance of any extramedullary sites. The results were expressed by means of leukemia-free survival (LFS), calculated from the date of achieving remission to that of the last follow-up, relapse or second malignancy. Nonresponders were classified as LFS=0. Patients undergoing hematopoietic stem cell transplantation were censored at the time of transplantation.

Statistical analysis. The Mann-Whitney *U*-test was used for unpaired comparisons. Survival curves were calculated by the Kaplan-Meier method and compared by log-rank test. Patients who received stem cell transplantation were censored at the time of transplantation. The Cox proportional hazards regression model was used to test for correlation of each potential prognostic factor with survival in univariate analysis. The factors significantly important (shown in Table I) were fitted together in multivariate analysis in a backward stepwise manner using the likelihood ratio test until all factors in the model were significant. All reported *p*-values are two-sided; $p<0.05$ was considered as statistically significant.

Results

The probability of overall survival for the whole group was 0.57±0.05, while pLFS=0.58±0.06, and mean survival was 3.36 years (95% confidence interval (CI)=2.95-3.77). Children who relapsed during follow-up exhibited a non-significant higher median *in vitro* resistance of leukemic blasts to most of the drugs tested, except for cytarabine, cladribine, vincristine, mercaptopurine and thioguanine. pLFS was not significantly better in patients with myeloblasts sensitive *in vitro* to 4-HOO-cyclophosphamide (0.83±0.15 *vs*. 0.65±0.09, p=0.12), doxorubicin (0.81±0.12 *vs*. 0.64±0.09, p=0.18), epirubicin (0.72±0.13 *vs*. 0.61±0.12, p=0.26), fludarabine (0.73±0.12 *vs*. 0.62±0.11, p=0.22), mitoxantrone (0.77±0.12 *vs*. 0.51±0.13, p=0.07), treosulfan (0.88±0.12 *vs*. 0.62±0.11, p=0.29), and etoposide (0.70±0.13 *vs*. 0.63±0.09, p=0.4).

A combined *in vitro* fludarabine, treosulfan and mitoxantrone resistance profile (FTM score) proved to be discriminative for children with AML in univariate analysis of pLFS: patients with leukemic cells classified as sensitive according to their FTM score had a significantly better pLFS than those with myeloblasts resistant to fludarabine, treosulfan and mitoxantrone (0.73±0.12 *vs*. 0.50±0.14, p=0.034) (Figure 1). The results of other risk factors are shown in Table I. Factors significant by univariate analysis were fitted together in a Cox model of multivariate analysis. Two factors showed prognostic value: early BM response by day 15 (p=0.021; HR=0.29, 95% CI=0.13-0.64) and myeloblast sensitivity as determined by the combined *in vitro* fludarabine, treosulfan and mitoxantrone resistance profile (FTM score), p=0.048; HR=0.38, 95% CI=0.14-0.97.

Discussion

One of the key factors in success of antileukemic therapy is related to significant progress in stratification and risk-adapted therapy. Growing evidence supports the thesis of the prognostic significance of the *in vitro* drug resistance profile in childhood ALL (9, 10, 23), but no relevant data have been obtained in pediatric AML to date. Early reports have suggested the putative prognostic role of *in vitro* resistance to cytarabine (15), but these data were not confirmed in subsequent analyses. We have shown in this study that the combined *in vitro* fludarabine, treosulfan and mitoxantrone resistance profile has a strong prognostic significance in childhood AML. We have shown the impact of the *in vitro* drug resistance of 3 agents belonging to 3 different classes of drugs: the nucleoside analogue fludarabine, the topoisomerase II inhibitor mitoxantrone and the alkylating agent treosulfan. These three groups of drugs play a key role in the therapy of childhood AML, however fludarabine, treosulfan and mitoxantrone are relatively rarely used in clinical practice in pediatric oncology.

Drug resistance profiles identified patients at higher risk of treatment failures. The combined *in vitro* drug resistance profile was one of the strongest prognostic factors in a multivariate analysis for AML of children. No firm suggestions regarding the use of fludarabine, treosulfan and mitoxantrone in the treatment of AML can be drawn from our study, however these results might indicate that patients whose myeloblasts were sensitive to fludarabine, treosulfan and mitoxantrone would possibly benefit from the use of these drugs during the early stages of therapy. Patients resistant to these drugs should be considered as a high-risk group or given tailored therapy (24). In spite of the prognostic significance of *ex vivo* cytotoxicity results, cytogenetics of myeloblasts still has a very strong predictive value in pediatric AML (25).

In conclusion, the *in vitro* combined fludarabine, treosulfan and mitoxantrone resistance profile to is of predictive value in childhood AML. Therefore, the drug resistance profile may be used for better stratification of children with AML to prevent overtreatment of those patients who may be cured by relatively mild chemotherapy and to identify those patients who are at high risk of treatment failure and who, therefore, may benefit from more intensive treatment at initial diagnosis.

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